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THE IMPLICATION OF CARBONIC ANHYDRASE IN THE PHYSIOLOGICAL MECHANISM OF PENETRATION OF CARBONATE SUBSTRATA BY THE MARINE BURROWING SPONGE *CLIONA CELATA* (DEMOSPONGIAE)

WALTER I. HATCH

Department of Biology, Southeastern Massachusetts University, North Dartmouth, Massachusetts 02747

Marine sponges of the family Clionidae (Gray, 1867) are structurally and functionally similar to other members of the class Demospongiae, phylum Porifera (Vosmaer, 1933–1935; Hyman, 1940). They are, however, unique among the sponges in that each species in the family has the ability to excavate a habitation, in the form of dendritic or anastomosing galleries, in a variety of calcareous substrata (Hancock, 1849; Leidy, 1889).

In clionid sponges most of the tissues lie completely enclosed within the calcareous substratum, with the exception of numerous papillae which extend to the surface through holes in the substratum. Several ostial pores open into each incurrent papilla, giving them the appearnce of a sieve plate, while each excurrent papilla terminates in a single large osculum. Both types of papillae are strongly contractile and enable the sponge to insulate itself from unfavorable environmental conditions (Emson, 1966).

As the sponge grows, the galleries are enlarged and new channels established by the removal of numerous small chips, which are transported to the surface through the excurrent canal system in a manner analogous to a mining operation (Nassonow, 1883; Topsent, 1888; Cobb, 1969, 1971).

In many clionids the galleries remain discrete and the sponge does not completely destroy the substratum. In *Cliona celata,* however, the sponge tissue spreads from the base of the papillae to cover the surface of the substratum, continually removing carbonate until the galleries fuse and the substratum is completely eliminated, leaving an unenclosed free-living sponge. Although this is a continuous process, for convenience it has been divided into three stages: alpha stage while the sponge is completely enclosed, grading into beta stage as the sponge extends across the surface, and finally gamma stage when the substratum has been completely destroyed (Vosmaer, 1933–1935).

The nature of the mechanism employed by the Clionidae to excavate calcareous substrata has been the subject of investigation, speculation, and controversy since 1826, when Osler first described a sponge-like organism within the valves of oysters (Osler, 1826). Three major hypotheses on the mode of penetration have been presented: an extensive chemical dissolution of the substratum, usually assumed to be the action of some acidic etching agent; an exclusively mechanical removal of the substratum, and a chemomechanical mechanism in which the substratum is chemically softened or loosened and subsequently mechanically removed. The most tenable mechanism is the combination of a localized chemical dissolution coupled with mechanical dislodging of fragments of the substratum and their subsequent transport out of the sponge galleries.

It seems evident from the manner in which chips are freed from the substratum (Cobb, 1969, 1971; Rutzler and Reiger, 1973) and from the fact that calcium carbonate is the primary mineral excavated by burrowing sponges, that the mechanism of excavation involves a localized modification of the calcium carbonate solubility equilibrium. Little progress has been made to date in the identification of an etching agent with this capability.

The potential difficulties of investigating penetration mechanisms that function at the cellular level suggest that a direct analysis of the clionid etching agent is not feasible at this time. This investigation was, therefore, undertaken not to demonstrate the presence of an acidic etching agent in *Cliona celata,* but rather to demonstrate the physiological capability of the sponge to produce such an agent or otherwise alter ion concentration products, thus affecting solubility.

The participation of the enzyme carbonic anhydrase in shifting solubility equilibria, resulting in the deposition of calcium carbonate by invertebrates, is well established (Goreau and Hartman, 1963; Heatfield, 1970; Istin and Girard, 1970). Carbonic anhydrase has been implicated in the dissolution of carbonate as well. The enzyme has been found in the accessory boring organ of shell-boring muricid gastropods (Chétail *et al.*, 1968; Smarsh *et al.*, 1969) and its activity has been related to the process of excavating calcium carbonate substrata by acrothoracican cirripeds (Turquier, 1968) and muricid gastropods (Chétail and Fournié, 1969; Carriker and Chauncey, 1974; Rosenberg *et al.,* 1968).

Thus, there is ample evidence that carbonic anhydrase operates in the dissolution and deposition of calcium carbonate in a variety of systems. The purpose of this investigation is to demonstrate this enzyme in actively excavating *Cliona celata* Grant, and its possible involvement in penetration of calcium carbonate by the sponge.

Materials and Methods

Assay for clionid carbonic anhydrase

Carbonic anhydrase was assayed by an electrometric method (Wilbur and Anderson, 1948; Davis, 1963; Carter *et al.,* 1969; Carter, 1972).

Buffer preparation. Tris buffer (0.025 M, pH 8.8 at 0°C) was prepared by dilution of a 0.25 M stock solution immediately before use. The dilute buffer was protected from CO_2 contamination.

Substrate preparation. A saturated solution was prepared by bubbling pure CO_2 through deionized glass-distilled water, at 0°C, for at least 1 hr prior to use. The resulting substrate solution was 0.076 M CO_2, resulting in a final substrate concentration of 29.42 mM when a 2.50-ml aliquot was added to the 6-ml reaction mixture.

Enzyme preparation. Alpha-, beta-, and gamma-form specimens of *Cliona celata* from the Northwest Gutter of Hadley's Harbor, Naushon Island, Massachusetts, were maintained in running sea water. The sponges were identified by spicule examination (Old, 1941). Sponge tissue was obtained by drilling a 10-mm hole in the apex of shells in which alpha- and beta-form sponges were burrowing or by removing a plug of tissue from gamma-form sponges. This material was freed of shell fragments and sponge tissue by treatment with warm concentrated nitric acid, rinsed twice with distilled water and once with 95% ethanol, and spread on a slide for microscopic examination.

In preparation for enzyme extraction, living sponges were cleaned of epibiota by scraping and scrubbing briefly with a bottle brush and tap water. A sponge cell suspension was produced by manually expressing excess water, fragmenting with a hammer when necessary, and forcing the fragmented sponge tissue through a 2-mm stainless-steel sieve to remove inorganic inclusions and shell debris. Enzyme solutions were prepared by disrupting the cell suspension with a Waring blender. After dialysis (24 hr, distilled water at 0°C), enzyme solutions were diluted to 1–2 mg total protein/ml.

In order to eliminate the effects of non-catalytic protein buffering (Addink, 1971), controls were run in the presence of 10^{-3} M acetazolamide resulting in complete inhibition of enzyme activity. Inhibited enzyme solutions were prepared by adding 3.3 mg sodium acetazolamide to a 2.5 ml aliquot of dilute enzyme solution and titrating back to the pH of the uninhibited enzyme solution (7.25) with HCl.

Assay apparatus and procedure. The enzyme assay apparatus differed from that of Carter *et al.* (1969) in that the reaction vessel, delivery syringes, and reservoirs for buffer and substrate storage were all housed in a water bath thermostatically regulated to 0± 0.1°C. In addition, provision was made for transferring substrate solutions under positive pressure to avoid degassing and the resulting fluctuations in substrate concentration. A magnetic stirring device insured rapid mixing.

In use, 2.5 ml of buffer was transferred from delivery syringe to reaction vessel and 1.0 ml of either enzyme or control solution added. After 30–60 sec for electrode equilibration, 2.5 ml of substrate solution was injected and a chart recorder started simultaneously. The reaction was allowed to proceed to equilibrium before the reaction vessel was drained and flushed for the next determination.

The initial rate of catalyzed and control reactions was determined by fitting a tangent to the [H^+]-time curve at pH 8.70. From these tangents Δ pH/min could be determined and converted to mM H^+/min from a buffer-enzyme calibration curve. The enzymatic rate was determined by substracting mM H^+/min produced in control reactions from that produced in enzyme catalyzed reactions. The mean of triplicate runs was recorded as enzyme activity.

Intracellular localization of clionid carbonic anhydrase

Clionid cell suspensions were disrupted in 0.025 M sucrose at 0°C by alternating 30 sec of grinding with 5 min of cooling (4 ×) in a Waring blender. The resulting homogenate was centrifuged (10 min, 500 × *g*) to remove inorganic inclusions and unbroken cells. The supernatant was recentrifuged (10 min, 700 × *g*) over a layer of 0.34 M sucrose to sediment nuclei. The supernatant was again collected and recentrifuged (10 min, 21,000 × *g*) to sediment mitochondria-like particles. Each pellet was resuspended in distilled water and, along with the supernatant, dialyzed against stirred distilled water for 24 hr at 0°C. Each retentate was then assayed for carbonic anhydrase activity.

An aliquot of the supernatant (10 min, 700 × *g*) of a second sucrose homogenate was centrifuged at 21,000 × *g* for 15 min. A second aliquot was spun (21,000 × *g*) for 60 min. Third and fourth aliquots were sonicated for 15 and 30 min, respectively, prior to centrifugation (21,000 × *g*, 60 min). The fifth aliquot was treated with N-butanol (20%, 1 hr, 0°C) according to the standard technique for butanol treatment outlined by Morton (1955). The butanol treated aliquot was also centrifuged for 1 hr at 21,000 × *g*, and the aqueous and butanol phases

separated. The supernatants thus produced were all dialyzed against distilled water, diluted to a constant volume, and assayed for carbonic anhydrase activity.

Excavation rate and carbonic anhydrase content of clionid sponges

If clionid carbonic anhydrase is involved in the physiological mechanism of excavation, it is probable that the excavation rate is related to the concentration of the enzyme in the sponge. The difficulties involved in determining the excavation rate of clionid sponges were circumvented by developing an alternative technique (Hatch, 1974). In that as much as 90% or more of the substratum is excavated in the form of carbonate chips (Warburton, 1958), the weight of the chips expelled per unit time was used as an estimate of excavation rate.

Procedure. Alpha-, beta-, and gamma-form specimens of *Cliona celata* were cleaned of epibiota, positively identified as previously described, and placed in petri dishes in a flowing sea-water table. Each day expelled chips were collected on Whatman #4 filter paper, rinsed with distilled water and air dried for determination of calcium carbonate weight. The excavation rate was recorded as mg $CaCO_3$ expelled per day (averaged over 4 days), and the sponges were divided into actively excavating (25–50 mg $CaCO_3$/day), slowly excavating (1–25 mg $CaCO_3$/day), and non-excavating (no detectable carbonate chips). Four specimens from each group were extracted with butanol as previously described and assayed for carbonic anhydrase activity. In addition, gamma-form cortical and medullary tissues were extracted and assayed separately.

Effects of carbonic anhydrase inhibition on excavation rate

In order to establish a possible relationship between the physiological mechanism of penetration and carbonic anhydrase, the excavation rate of *Cliona celata* was determined in the presence of acetazolamide, a specific inhibitor of this enzyme.

Procedure. Thirty actively excavating alpha-form specimens of *Cliona celata* within *Mercenaria mercenaria* valves were freed of epibiota, identified, and returned to a flowing sea-water table as previously described. Through visual inspection of chip production over the course of a week, the 10 most active sponges were selected and arranged convex side up in petri dishes for chip collection (Hatch, 1974).

An inhibitor stock solution was prepared by dissolving sodium acetazolamide in Millipore-filtered sea water (1×10^{-4} M) and adjusting the pH back to that of the sea water system (8.26) with HCl. Inhibitor solution was introduced into flowing sea water through a metered-drip intravenous-infusion apparatus at a rate sufficient to maintain the desired acetazolamide concentration.

The excavation rate was determined over two 24 hr periods under the following conditions: 2.5 l/min sea water flow-through, 0.25 l/min flow-through with 2.25 l/min recirculation, and 0.25 l/min flow-through with 2.25 l/min recirculation in the presence of 10^{-5} and 10^{-6} M acetazolamide. The recirculation was necessitated by the sponges' requirement for a minimum current velocity and economic constraint on producing a 10^{-4} M inhibitor concentration with a large flow-through.

At the start of each determination sponges were carefully transferred under water into clean petri dishes. Expelled chips were collected after 24 hr as previously described.

Effect of carbonic anhydrase inhibition on in vitro metabolic rate

The possibility of toxic secondary effects of acetazolamide on the excavating ability of *Cliona celata* was investigated with *in vitro* respirometry utilizing a Gilson differential respirometer.

Procedure. The medullary tissue of beta-form specimens of *Cliona celata* was dissected from between the attached closed valves of *Mercenaria mercenaria.* The tissue was pooled, rinsed in running sea water, and teased into fragments less than 1 mm on a side.

Approximately 1 cm^3 of this tissue was placed in each Gilson reaction flask along with 3 ml of Millipore-filtered sea water. One half of one ml of a 20% w/v KOH solution was used as the CO_2 absorbant. One milliliter of the inhibitor solution (8.8×10^{-3} or 8.8×10^{-4} mg sodium acetazolamide/ml Millipore-filtered sea water, pH 8.26) was added to the side arm of each reaction flask. Fourteen replicates of each inhibitor concentration were run.

The flasks were equilibrated for 30 min, after which six readings were taken at 5-min intervals to establish the control respiratory rate. The inhibitor solution was then tipped into the sea water containing the sponge tissue and the O_2 consumption in the presence of 10^{-5} and 10^{-6} M acetazolamide was established with six additional readings at 5-min intervals. The total protein concentration of each flask was then determined by UV absorbance (Warburg and Christian, 1942).

Effects of carbonic anhydrase inhibition on papillary contraction

The ability of clonid sponges to respond to chemical sitimuli with papillary contraction has been documented (Emson, 1966). It is likely that papillary contraction and the resulting restriction of ostial and oscular openings could indirectly inhibit excavation by limiting the flow of sea water through the sponge. *In vivo* polarographic respirometry was utilized to reflect the degree of papillary contraction elicited by sodium acetazolamide.

Procedure. Alpha-form specimens of *Cliona celata* contained within *Mercenaria mercenaria* valves were selected to provide the maximum amount of respiring tissue that could be isolated by papillary contraction. Three individual valves were cleaned of epibiota and pooled for each experimental determination.

The oxygen consumption of the sponge was determined in a flow-through system in which sea water (1000 ± 2 ml/min) was passed first over the sponges in a sealed lucite chamber and then over a polarographic oxygen electrode coupled to a strip-chart recorder. The control respiratory rate was recorded for 30 min, after which an inhibitor stock solution (10^{-3} M sodium acetazolamide in sea water) was introduced into the inlet flow at 1 ml/min for an additional 30-min period. The inhibitor stock solution was then increased to 10^{-2} M and a final 30-min record of the respiratory rate was obtained.

Papillae were then induced to contract in response to a mechanical stimulus (tapping the lucite chamber), checking the function of the apparatus and the responsiveness of the sponges. Three replicates were run in this manner at 17.5° C. Oxygen consumption, indicative of the state of papillary contraction, was calculated from the difference in O_2 saturation of the sea water before and after it had passed over the sponges.

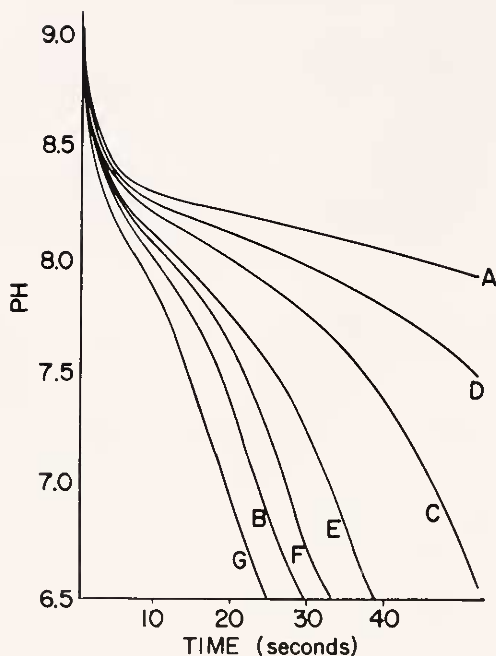

FIGURE 1. Clionid carbonic anhydrase reaction curves. Changes in pH are plotted against time for: A—distilled water control, B—sucrose extract prior to centrifugation, C—supernatant of sucrose extraction after centrifugation for 15 min at $21{,}000 \times g$, D—supernatant of sucrose extraction after centrifugation for 60 min at $21{,}000 \times g$, E—supernatant of sucrose extraction with 15 min of sonication prior to centrifugation (60 min, $21{,}000 \times g$), F—supernatant of sucrose extraction with 30 min of sonication prior to centrifugation (60 min, $21{,}000 \times g$), G—supernatant of sucrose extraction after treatment with N-butanol prior to centrifugation (60 min, $21{,}000 \times g$). Each line represents the mean of three runs.

RESULTS

Carbonic anhydrase activity was evident in crude water extracts. This activity was, however, largely removed by centrifugation, suggesting that the enzyme was not in cytoplasmic solution. The majority of enzyme activity was found in the precipitate from centrifugation at $21{,}000 \times g$. Spinning for 60 min removed more activity from the supernatant than spinning for 15 min. Sonication prior to centrifugation reduced the loss of activity from the supernatant. Finally, treatment with butanol precluded loss of enzyme activity from the supernatant during centrifugation even at $48{,}000 \times g$ for several hours. The butanol phase contained no measurable carbonic anhydrase activity.

Excavation rate and carbonic anhydrase content

The enzymatic rate from the assay of each sponge is shown in Table I as enzyme units/mg of protein assayed. As can be seen from the data, there is a tendency for the more rapidly excavating alpha-form sponges to contain a higher concentration of carbonic anhydrase. The difference between the 25 and 50 mg/day group and the non-excavating group is of marginal significance (Student's t-test, $t = 1.16$, $0.10 < P < 0.15$). The difference between the carbonic anhydrase activities of the rapidly excavating sponges and gamma form medullary tissues was, however, highly significant (Student's t-test, $t = 9.01$, $P < 0.005$).

TABLE I

Carbonic Anhydrase content of alpha, beta, and gamma forms of Cliona celata.

	Sponge #	Milligrams protein/ml	E.U./mg*	Mean	s.e.
Alpha form	1	1.93	3.1		
0 mg $CaCO_3$	2	1.87	26.1	21.28	3.55
excavated/day	3	1.96	29.9		
	4	1.79	26.0		
Alpha form	5	0.75	30.5		
1–25 mg $CaCO_3$	6	1.31	31.8	24.00	2.40
excavated/day	7	1.96	16.2		
	8	1.70	17.5		
Alpha form	9	1.83	22.9		
25–50 mg $CaCO_3$	10	1.93	30.4	25.49	1.00
excavated/day	11	1.82	23.3		
	12	2.00	25.1		
Beta form	13	1.85	11.0		
0 mg $CaCO_3$	14	1.83	28.3	20.00	4.41
excavated/day	15	1.83	3.8		
	16	1.89	36.9		
Gamma form	17	1.79	37.6		
cortex	18	1.80	36.8	38.83	0.70
0 mg $CaCO_3$	19	1.91	39.1		
excavated/day	20	1.61	41.8		
Gamma form	21	1.89	16.6		
medulla	22	1.87	13.4	14.13	1.42
0 mg $CaCO_3$	23	2.01	11.3		
excavated/day	24	1.57	15.2		

* E.U. (enzyme unit) = 1 μM H^+/min.

Effects of carbonic anhydrase inhibition on excavation rate

The effects of enzyme inhibition on excavation rate are presented in Table II. There was considerable variability in the amount of carbonate excavated by the sponges during the two determinations. In spite of this variability, there was a profound effect on the excavation rate in the presence of carbonic anhydrase inhibition. In the presence of a 2.5 l/min flow-through, the average rate was 44.6 ± 4.8 mg of $CaCO_3$/day. When the flow-through was reduced to 0.25 l/min and the water was recirculated at 2.25 l/min, there was a slight, statistically insignificant decrease in the excavation rate to 41.2 ± 5.2 mg of $CaCO_3$. These results indicate that the reduced flow-through rates necessary to maintain the required inhibitor concentration may lower the excavation rate slightly, even when the current across the sponges is augmented by recirculating the sea water. In the presence of 10^{-6} M acetazolamide, however, there was a profound and statistically significant decrease in the excavation rate (13.6 ± 5.2 mg $CaCO_3$/day per sponge). In the presence of 10^{-5} M acetazolamide the excavation rate was further reduced to 3.0 ± 1.0 mg $CaCO_3$/day for each sponge. The total weight of the carbonate excavated by all of the sponges in each experiment demonstrates the same marked effect of the inhibitor on the excavation rate. The controls produced 823 mg $CaCO_3$/day while the same sponges in the presence of 10^{-6} and

TABLE II

Excavation rate of Cliona celata *in the presence of carbonic anhydrase inhibition. (Experimental condition: A = 2.50 l/min fresh sea water through-flow, B = 0.25 l/min fresh sea water through-flow augmented with recirculation (2.25 l/min) to an apparent flow of 2.50 l/min, C = as B with sodium acetazolamide added to a final concentration of 10^{-5} M, D = as B with 10^{-6} M acetazolamide.*

Experimental condition	Milligrams of calcium carbonate removed per day per sponge												
	Run one										$\bar{X}$	s.e.	Σ
A	41	60	4	61	34	39	71	72	53	59	49.4	6.5	494
B	54	18	36	99	46	18	90	84	11	66	43.2	9.4	432
C	00	00	02	01	03	04	05	05	06	07	03.3	0.78	033
D	16	17	12	19	10	08	16	12	11	13	13.4	1.1	134
	Run two												
A	55	44	34	24	33	00	73	63	17	56	39.9	7.1	399
B	40	20	23	31	37	37	43	73	32	53	39.1	4.9	389
C	00	01	01	02	00	07	05	04	07	00	0.27	0.89	027
D	18	19	15	19	08	05	05	21	14	14	13.8	1.7	138

Experimental condition	Total	Mean	Standard error	Number of specimens
A	893 mg	44.6 mg	4.8 mg	20
B	823 mg	41.2 mg	5.2 mg	20
C	060 mg	03.0 mg	1.0 mg	20
D	272 mg	13.6 mg	5.2 mg	20

10^{-5} M acetazolamide produced only 272 and 60 mg of calcium carbonate, respectively.

From these data it can be seen that the presence of acetazolamide does, in fact, inhibit the ability of *Cliona celata* to excavate calcium carbonate from the valves of *Mercenaria mercenaria.*

Effects of carbonic anhydrase inhibition on in vitro *metabolic rate*

The *in vitro* metabolic rate of the sponges remained unchanged by the addition of acetazolamide. The mean respiratory rate, in mm^3 O_2 consumed/100 mg of soluble protein, is shown graphed against time in Figure 2. From this graph it can be seen that the difference in metabolic rate in the presence and absence of acetazolamide falls within the standard error of the measurements. Thus, the effect of 10^{-5} and 10^{-6} M acetazolamide on the excavating ability of *Cliona celata* cannot be attributed to an inhibition of the sponge's metabolism.

Effects of carbonic anhydrase inhibition on papillary contraction

The *in vivo* metabolic rate was also unaffected by acetazolamide. Figure 3 shows oxygen consumption of the sponges (in μl O_2/min.) in the presence of acetazolamide. From this figure it can be seen that several minutes are required for the sponge to re-expand its papillae and resume normal oxygen consumption

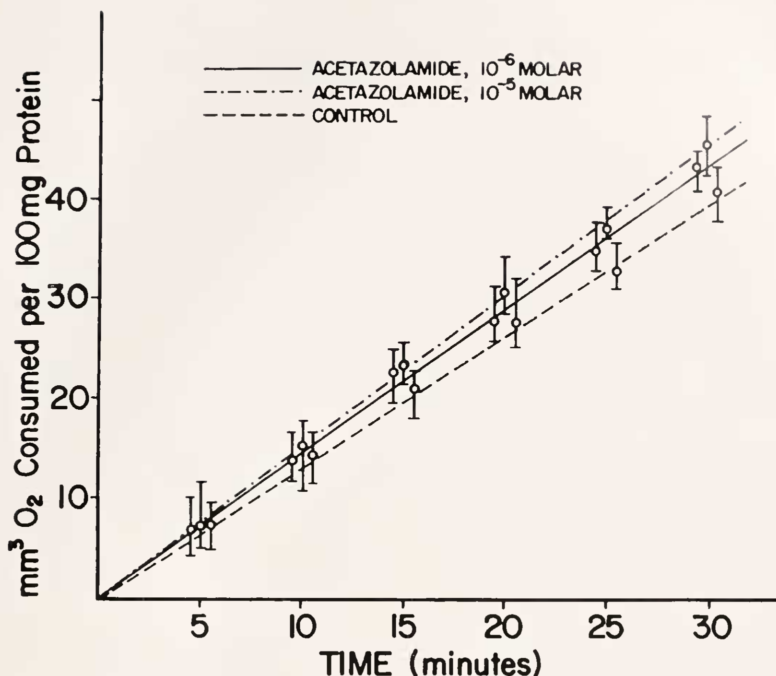

FIGURE 2. The effects of carbonic anhydrase inhibition on *in vitro* basal metabolism.

after handling. The small peaks just before T_0 may represent an increase in oxygen consumption resulting from an oxygen debt incurred during the handling period. No apparent difference in oxygen consumption can be seen from these graphs, indicating no detectable papillary contraction occurs in the presence of the concentrations of acetazolamide used to inhibit excavation. The mechanical tap, however, produces a dramatic change in oxygen consumption as the papillae contract in response to this stimulus.

Visual observation during the course of the experiment confirms that no papil-

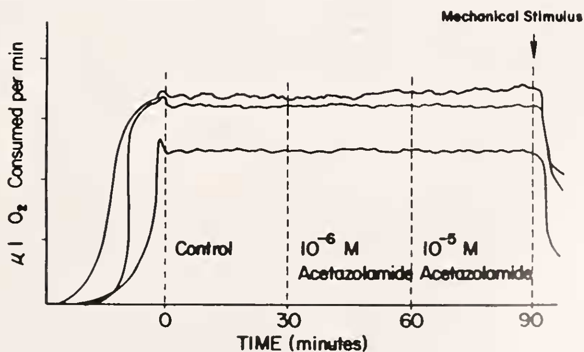

FIGURE 3. The effects of carbonic anhydrase inhibition on papillary contraction and *in vitro* respiratory rate. Respiratory rate of three pooled sponges is shown for three experimental runs. (Baseline rate established for 30 min prior to the addition of 10^{-6} and 10^{-5} M acetazolamide.) The mechanical stimulus served as an equipment check (note rapid drop in respiratory rate with papillary contraction).

lary contraction occurs in response to acetazolamide. It can then be concluded that the inhibition of clionid excavation by acetazolamide is not mediated by the ability of the sponge to detect and respond to inhibition by papillary contraction.

Discussion

The exact manner by which carbonic anhydrase might mediate the finely controlled dissolution of the substratum by clionid etching cells is unclear. Unlike the carbonic anhydrase of *Urosalpinx cinerea,* which is in cytoplasmic solution in the microvillar zone of the accessory boring organ (Smarsh *et al.,* 1969) or released in the ABO secretion (Carriker and Chauncey, 1974), clionid carbonic anhydrase appears to be associated with mitochondrial-sized particles.

The presence of carbonic anhydrase in the tissues of *Cliona celata,* however, indicates that the sponge has the capacity to greatly increase the speed of the reversible reaction $H_2O + CO_2 \rightleftharpoons H^+ + HCO_3^-$ and accelerate the precipitation or dissolution of $CaCO_3$ by shifting the solubility equilibria. Both the tendency for rapidly excavating sponges to have higher carbonic anhydrase activities than non-excavating sponges and the inhibition of excavation rate by specific enzyme inhibitors strongly suggest the participation of this enzyme in the excavation process.

The enzyme concentration of gamma-form sponges presents a problem. Although no substratum was present, the carbonic anhydrase concentration in the cortical tissues was much higher than in alpha- or beta-form sponges. The fact that the cortical tissues contained much higher concentrations of the enzyme than the medullary tissues suggests the enzyme may be localized in surface-lining cells. It is these cells that would come in contact with fresh substratum. The lower enzyme concentrations of beta-form sponges may also be attributed to a relative increase in non-excavating tissue.

Rutzler and Reiger (1973) demonstrated a prominent nucleolus, abundant ribosomes, and an extremely active golgi complex within the etching cells of clionid sponges, suggesting that these cells are involved in protein and carbohydrate synthesis even in advanced stages of plasmolysis. In addition, the filopodial basket contains numerous vesicles as well as a flocculent secretory product. Although they never saw these vesicles emptying their contents to the outside (Rutzler, personal communication), they speculated that the flocculent material observed by them within the etching cells, and in the space between the cell and substratum, is responsible for the penetrating activity of these cells.

It is unlikely, however, that the secretory product seen by Rutzler and Reiger represents carbonic anhydrase, as a direct catalytic breakdown of the substratum by the enzyme has been ruled out (Carriker and Chauncey, 1974). Clionid carbonic anhydrase activity can be removed from crude aqueous extracts by centrifugation, suggesting the enzyme might be associated with some subcellular structure (Morton, 1955). The enzyme could occur, therefore, in solution within a limiting semipermeable membrane (Schneider, 1953) or intimately associated with the insoluble lipoprotein of the membrane (Morton, 1953, 1954). The fact that sonic or butanol disruption of membranes precludes loss of enzyme activity during centrifugation supports the hypothesis that the enzyme is contained within, or is associated with, the membrane of vesicles within the filopodial basket.

There are several equally plausible mechanisms for the participation of carbonic anhydrase in the penetration of both organic and inorganic components of shell. First, carbonic anhydrase, within the filopodial basket, could accomplish the dis-

solution of calcium carbonate by simply providing hydrogen ions for transport across the membrane. Thus, the mechanism may be similar to that found in parietal cells (Maren, 1967) in which the H^+ ions are transported across the membrane along with Cl^- ions.

It is equally valid to speculate that the mechanism of penetration involves carbonic anhydrase in a role similar to that found in mammalian kidney (Maren, 1967). In clionid etching cells the exchange of hydrogen for bicarbonate ions would result in both the dissolution of the substratum and a lowering of the pH, with possible optimization for the enzymes responsible for the dissolution of the organic matrix.

In addition, carbonic anhydrase has been implicated in the transmembrane transport of Ca^{2+} ions (Istin and Kirschner, 1968; Ehrenspeck *et al.,* 1971). Thus, the penetration mechanism may involve a transmembrane flux of Ca^{2+} ions as well as a simple pH shift.

It is also possible that the H^+ ions resulting from the activity of carbonic anhydrase participate only indirectly in the dissolution of the substratum by providing pH optimization for the activity of chelating agents and/or the enzyme responsible for the breakdown of the organic matrix. The experimental determination of a chelating agent within the etching cell or its penetrative agent must be accomplished to confirm this hypothesis.

In summary, carbonic anhydrase in *Cliona celata,* coupled with the demonstration that the concentration of the enzyme in the sponge tissues is related to the excavating activity of the sponge, suggests this enzyme is involved in the physiological mechanism of penetration. This conclusion is supported by evidence that enzyme inhibition results in inhibition of the excavating ability of the sponge. The inability of the sponge to detect and respond to acetazolamide with papillary contraction or a depressed metabolic rate indicates that this inhibition of excavation rate is neither mediated by a behavioral response nor by a generalized metabolic inhibition.

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Summary

1. The marine burrowing sponge *Cliona celata* contains measurable carbonic anhydrase activity when assayed with a modified electrometric method.

2. When clionid tissues are extracted and centrifuged in isotonic sucrose, the majority of the enzyme activity is found in the mitochondrial size fraction, indicating the enzyme is membrane- or particle-bound.

3. Much of the enzyme activity can be released into solution with sonication. Treatment with isobutanol, which dissociates lipoprotein complexes, releases all of the enzyme activity into solution.

4. From a range of buffer solutions, 0.025 M trisaminomethane (pH 8.4–8.7) was chosen as the optimal buffer for the assay of clionid carbonic anhydrase.

5. There is a positive correlation between the excavating activity of the sponge (mg of $CaCO_3$ removed per day) and the level of carbonic anhydrase in sponge tissues. In addition, enzyme activity is concentrated in the cortical tissues of gamma-form *C. celata*. It is these tissues that are most likely to come in contact with fresh substrata in the form of shell fragments.

6. Sodium acetazolamide is capable of inhibiting the activity of clionid carbonic anhydrase *in vitro* with no apparent inhibition of the overall metabolism of the overall metabolism of the sponge.

7. Acetazolamide-induced inhibition of the sponge's carbonic anhydrase activity markedly reduces the ability of alpha-form *C. celata* to excavate calcium carbonate chips from the valves of *Mercenaria mercenaria*.

8. Similar concentrations of acetazolamide provoke no papillary contraction or reduction in oxygen consumption *in vivo*, indicating the reduction in excavation rate is not due to the ability of the sponge to detect the acetazolamide and shut down the flow of sea water through its choanosome. It appears, therefore, that the primary mechanism for the dissolution of calcium carbonate by *C. celata* involves a shift in the carbonate solubility product in the microenvironment of the etching cell, mediated through the activity of the enzyme carbonic anhydrase.

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